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PROVISIONAL APPLICATION FOR PATENT COVER SHEET

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INVENTOR(S)/APPLICANTS(S)

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TITLE OF THE INVENTION (280 character maximum)

Immunostimulation Through Activation of Phagocytic Cells

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Respectfully submitted,

SIGNATURE: _____ Date: December 7, 2004

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MBHB 04-997

Immunostimulation through activation of phagocytic cells

Design, preparation and utility of a peptide mimetic of protein-bound N-acetylgalactosamine that simulates the action of macrophage activating factor for therapeutic use for cancers and other diseases.

Abstract

A phage display library was screened with lectins to identify amino acid sequences that mimic a protein-bound N-acetylgalactosamine residue, which is required for activity of the serum macrophage activating factor. Peptide mimetics were synthesized from these sequences and found to stimulate phagocytic activity of adherent cells from peripheral blood. Administration of a peptide mimetic to canine patients with advanced cancer extended life beyond expectation and improved quality of life. The peptide mimetics are to be used for immune enhancement and extension of remission from cancer.

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Description

FIELD OF THE INVENTION

The invention relates to potent agents that stimulate activity of phagocytes such as macrophages and neutrophils in the blood. These agents can be used as therapeutic tools for various cancers and other diseases against which the immune system is effective.

SUMMARY OF THE INVENTION

The serum vitamin D-binding glycoprotein can be processed endogenously or exogenously to obtain a potent macrophage activating factor (MAF), which bears a required sugar, N-acetylgalactosamine (GalNAc). Inability to process the protein to the active form or enzymatic removal of this sugar *in vivo* results in immunosuppression. We identified amino acid sequences that mimic protein-bound GalNAc. Peptide mimetics of MAF that were synthesized according to this identification were effective in stimulating phagocytic activity of adherent cells from blood. These peptides are also effective therapeutically and have the advantage of providing no opportunity for inactivation by deglycosylation.

BACKGROUND OF THE INVENTION

Phagocytes such as macrophages and neutrophils provide a primary line of defense against a variety of diseases, including those caused by infectious agents and cancers (Gomme and Bertolini, 2004). During a study of the role of inflammation in development of immunity, Yamamoto and Homma (1991) discovered that a serum protein was required to activate macrophages. This protein is the vitamin D-binding protein (DBP), which is also called group specific component, Gc. DBP is an abundant, multifunctional glycoprotein in the serum. Highly conserved homologs of this protein occur among all mammalian species (Yang et al., 1990; White and Cooke, 2000). As its name implies, one role of the protein is as a vehicle for circulating vitamin D in blood. Another function involves binding of actin released into the blood during tissue injury. Most pertinent to this invention, the glycan of the serum protein can be processed to a potent anti-cancer agent, which is expressed through its macrophage activation and anti-angiogenesis activities (Kanda et al., 2002; Gomme and Bertolini, 2004).

The 51 kDa DBP consists of three major domains similar to albumin (Head et al., 2002; Otterbein et al., 2002). DBP is a glycoprotein that carries a single trisaccharide group (Yang et al., 1985; Cooke and David, 1985). The O-linked glycan is found in the carboxyterminal DomainIII, attached to the hydroxyl group of a specific threonine residue (Thr420 in protein from human). Its structure has been determined as NeuNAc(α2→ 3) Gal(ß1→ 3) GalNAc(α1→ O) Thr, with significant amounts of the O-glycan found only on the Gc1 isoform (Coppenhaver et al., 1983; Viau et al., 1983). Some of the glycans contain a second NeuNAc linked α2→ 6 to GalNAc. Extensive work by Yamamoto and colleagues (Yamamoto and Kumashiro, 1993; Yamamoto and Naraparaju, 1996a,b) suggested that DBP has remarkable therapeutic value as an activator of macrophages. Its potent stimulatory activity for macrophage phagocytosis is expressed when its glycoslyated site is processed to a single O-linked GalNAc by removal of the NeuNAc (sialic acid) and the Gal residues (Yamamoto and Homma, 1991; Yamamoto and Kumashiro, 1993). The protein can be processed to the active form *in vitro* by treatment with immobilized sialidase and ß-galactosidase (Yamamoto and Kumashiro, 1993; Yamamoto and Naraparaju, 1998). In animals, the modified protein, referred to as DBP-MAF, reduces tumor cell load (Kisker et al., 2003; Onizuka et al.,

2004), provides a therapy against viral infections such as HIV (Yamamoto et al., 1995), and promotes bone growth (Schneider et al., 1995; 2003). DBP-MAF has also been found to be an effective anti-angiogenesis factor (Kanda et al., 2002; Kisker et al., 2003). A lectin receptor that specifically binds GalNAc residues was identified on the surface of human macrophages (Iida et al., 1999).

Cancer cells secrete, and some virus particles carry on their surface, an enzymatic activity (N-acetylgalactosaminidase) that depeletes the precursor protein in the serum by removing the O-glycoside, which renders the protein inactive as a macrophage activating factor (Yamamoto et al., 1996, 1997). A decrease of this activity may be a major factor in progression of disease. Introduction of the *in vitro* processed protein leads to dramatic reduction in the amount of cancer cells in animals (Yamamoto and Naraparaju, 1997; Kanda et al., 2002; Kisker et al., 2003; Onizuka et al., 2004) and appears to also reduce the number of HIV particles in infected individuals (Yamamoto et al., 1995). This conclusion is based largely on the decrease in activity of N-acetylgalactosaminidase, whose level appears to be directly correlated with tumor and viral loads in cancer and in HIV-infected patients, respectively (Yamamoto et al., 1997).

Our invention provides a means to achieve a stimulant of phagocytic cells that is impervious to inactivation by the action of this enzyme. With the knowledge that DBP-MAF is active with the attached GalNAc and inactive when it is removed, and thus the likelihood that the macrophage receptor for MAF is a GalNAc-binding protein, bacteriophage-display libraries were screened for peptide sequences that mimic the protein-bound sugar. After sequencing DNA of phage particles, an amino acid sequence was identified, chemically synthesized, purified, and tested in cultures of adherent peripheral blood cells and by infusion into canine subjects with advanced cancers. The peptide was more effective in activating phagocytic cells than an equal weight of lipopolysaccharide, a potent stimulator of this activity. The peptide caused a rapid improvement in attitude and behavior and extension of life of end-stage canine cancer subjects. In a few cases, a decrease in tumor load was detected clinically.

The present invention provides processes for identifying amino acid sequences that mimic protein-bound N-acetylgalactosamine (GalNAc) by use of lectins, which include synthesizing linear or branched forms of the peptide mimetic and optionally attaching ligands or chromophores to such linear or branched form of the peptide identified for analysis of interaction with other molecules and/or determining the concentration of such peptides. In preferred embodiments, the invention provides compositions comprising a linear peptide mimetic of GalNAc, such as a peptide comprising or consisting of the amino acid sequences disclosed herein. Such compositions can comprise branched or linear peptides. By simulating the GalNAc-dependent activities of Gc-MAF, the peptide mimetics should have all the activities of the full-length protein. Therefore, these peptides should act as a stimulant of phagocytic activity of macrophages, neutrophils, or other phagocytes, and are thus useful in therapeutic methods including, but not limited to, cancer therapeutics, anti-viral therapeutics, anti-angiogenesis therapeutics, treatment of bone disorders, and as adjuvants for vaccinations.

DESCRIPTION OF THE METHODS FOR IDENTIFYING AND PREPARING MIMETIC OF GalNAc

Because little more than the sugar and a few amino acids show phenotypic macrophage activation (Schneider et al., 2003), we designed a peptide structure that provides activation but which cannot be inactivated by deglycosylation. Amino acid sequences were identified that would mimic protein-bound GalNAc by screening a phage display library by first selecting phage particles that bind to GalNAc-specific lectins and subsequent elution with 100 mM free GalNAc. An example of lectins that are useful in the screen is one purified from the snail *Helix pomatia*, which is highly specific for GalNAc (Hammerström and Kabat, 1971) and also binds specifically to the active form of Gc-MAF that contains GalNAc (Kanan et al., 2000). With the lectin as an analog of the receptor on macrophage cells, a peptide that binds to the lectin should mimic the activities of Gc-MAF.

Table 1 shows amino acid sequences that were derived from the lectin screen. Three phage libraries were used to generate these data, (1) phage with a 12-mer variable region (36 nucleotides), (2) phage with a 7-mer variable region flanked by cysteine residues to allow loop formation by disulfide bond formation and (3) phage particles with a 7-mer variable region (21 nucleotides). Phage particles that bound to the lectin and were subsequently eluted by competition with free GalNAc were replicated, and the DNA of each was sequenced to derive the amino acid sequences of the variable region. Figure 1 shows a flowchart description of the algorithm used to search for patterns among these groups of sequences. Table 2 provides an example of the process of analysis by the algorithm. A presentation of the results of this process is shown in Table 3 for all sequences collected thus far, when each of the amino acids is indicated by a functional subgroup designation (Enshell-Seijffers et al., 2003). The algorithm was developed to determine frequencies of functionally similar dimers, trimers and pentamers, for example. Those that appear most frequently comprise the core of the mimetic. A reduced number of sequences emerge after several rounds of enrichment by the lectin screen. Sequence analysis of DNA of a set of selected phage particles indicate that the method allowed identification of a consensus amino acid sequence (X1-QATQSNQHTPR, wherein X1 is V or another amino acid or polypeptide sequence), which is then validated against the most frequent pattern identified by the algorithm. The N-terminal "V" residue is preferred but not required. The consensus sequence was used to synthesize peptides (X1-QATQSNQHTPR-X2, wherein X2 is absent or is 1-5 amino acids of the sequence GGGSK), either in single (linear) or multi-valent (branched) forms (Figure 2). The "GGGS" sequence is a spacer that is present in the mutagenized protein in all phage particles (i.e., not part of the variable region). This spacer was retained in some embodiments of the peptides of the present invention to move the mimetic sequence away from the C-terminal core of the branched structure and kept in the linear sequence for consistency.

Multivalent structures were considered important because of the likelihood that activation requires cell-surface receptors to cluster. These peptides showed a stimulatory activity in

assays with blood cells that adhered to the surface of plastic microtiter plants, a characteristic of neutrophils and macrophages. Furthermore, infusion of the peptide into canine patients with several different cancers resulted in remarkable extension and improvement in the quality of life and in some cases a reduction in size of the primary tumor. No evidence was found for an immunogenic reaction against the peptide in the recipients after several months of treatment at the doses given, 10 to 200 nmoles per animal (dogs about 80 lb body weight). Thus, our chemical approach offers a major advance in the goal of achieving immunostimulant therapy.

In conjunction with a novel pattern recognition algorithm disclosed herein, sugar-specific mimetics can be used to develop a surface pattern recognition algorithm for sugar-binding receptors, using crystal structures of lectins that have defined the specificity of the sugar/peptide binding residues (Taroni et al., 2000). For example three loops of the GalNac specific lectin from *Robinia pseudoacacia* are conserved among lectins with the fourth loop conferring carbohydrate specificity (Rabijns et al., 2001).

The pattern-recognition algorithm disclosed herein is applicable to pattern recognition in any amino acid sequence and does not require an initial query sequence, unlike prior art methods. These pattern recognition techniques can be used to identify any pattern in amino acid sequences, as exemplified herein.

SYNTHESIS OF THE MIMETIC

The linear peptide mimetic (Figure 2) was synthesized with standard methodology utilizing Fmoc (9-fluorenylmethoxycarbonyl)-protected amino acids in a commercial continuous flow peptide synthesizer, with the sequence as VQATQSNQHTPRGGGSK. The peptide was synthesized with the C-terminal lysine (K) attached to the resin. Biotin was incorporated into the peptide with ϵ-biotinyl-lysine as the C-terminal amino acid, in which biotin is attached through amide linkage to the sidechain amino group of lysine. The biotin group provides a means to retrieve the peptide with associated proteins from reaction mixtures because of its high affinity with strepavidin to study interaction of the peptide with cellular components. Mass spectroscopy of the peptide product, purified by HPLC, detected a species with the correct predicted molecular weight (Figure 3).

The branched peptide (Figures 2 and 4) was synthesized, again by standard procedures with Fmoc-protected amino acids, in two stages. The C-terminal part of the peptide consisted of lysine(K)-βalanine(βA)-cysteine(C). Next, K was added to both the α- and ϵ-amino groups of K-βA-C to yield $(K)_2$K-βA-C, in which the α- and ϵ-amino groups of both terminal lysine residues are available for extension. The final product, therefore, is $[(VQATQSNQHTPRGGGS)_2K]_2K\beta AC$. A fluorophore is incorporated into this product by reaction with the thiol group on the C-terminal cysteine. The initial procedure involved dansylation using IAEDANS (Molecular Probes) (Figure 5) following a standard Molecular Probes protocol for thiol-reactive probes. The product is purified by HPLC, and the purity is monitored by mass spectrometry (Figure 4). The product is dried and then dissolved in sterile phosphate buffered saline, pH 7.4. Concentration is determined by absorbance of the fluorophore (extinction coefficient of this group, ϵ_{mM} =

5.7 cm^{-1}). A 1 mg/ml solution has an absorbance at 336 nm of 0.79. The product is stable for at least 3 months at 4°C and longer when frozen.

Identification of an amino acid sequence that mimics the sugar GalNAc allows synthesis of a "glycoprotein" analog of Gc-MAF in systems that do not perform glycosylation of proteins. For example, high levels of expression of proteins can be achieved in bacteria and in the chloroplast of algae and plants, systems that do not have the capacity to synthesize most glycoproteins. We synthesized genes for DomainIII of Gc-MAF with nucleotide sequences that are optimized for codon usage in the bacterium *Escherichia coli* and in the chloroplast of the model alga *Chlamydomonas reinhardtii*. Such constructs have the sugar mimetic placed within a larger carrier protein, which has the activity of DomainIII that was expressed in the baculoviral system, one that is capable of glycosylation (Yamamoto and Naraparaju, 1997). The use of the mimetic to replace the sugar greatly simplifies the production of active DomainIII or the full-length Gc-MAF in non-mammalian systems. Transformation of the chloroplast genome and the mechanism of expression of proteins within the chloroplast are similar to the processes in bacteria. *C. reinhardtii* chloroplasts are easily transformed by biolistic bombardment of cells with small gold beads that are covered with a vector DNA in which the gene of interest has been inserted. Figure 6 shows the amino acid sequence of the product. The synthetic gene also encodes C-terminal poly-histidine to allow affinity purification.

SUPPORTING DATA

1. Effect of peptide mimetic on cellular activity

    a) Oxidative burst

Peripheral blood samples are removed from animals and 300 µl added to wells of a 96-well microtiter plate and incubated overnight at 38°C in a standard CO_2-incubator. The nonadherent cells, including erythrocytes, and serum are removed and centrifuged to pellet cells and obtain cell-free serum. RPMI 1640 medium containing 2 to 10% serum from the same animal is added to each well and incubation of the culture is continued for various periods of time. The peptide mimetic is then added to a concentration of 1 to 10 nM and incubation continued for 3 h.

One type of assay of cellular activity measures the response of adherent cells to the peptide mimetic by the change in absorbance of cytochrome *c* (Johnston et al., 1978; Pick and Mizel, 1981). Phorbol 12-myristate 13-acetate (PMA) is added to 140 nM and then cytochrome *c* is added to 15 µM. Change in absorbance is monitored continuously at 550 nm over 20 min. With 300 µl in each well, reduction of cytochrome *c* was calculated as: Δnanomoles = absorbance at 550 nm x 100/2.1 (Pick and Mizel, 1981). Positive control samples are run with lipopolysaccharide, a known stimulator of macrophage activity. Negative controls lack stimulant. Figure 7 shows results of representative, reproducible experiments.

The oxidative burst upon addition of PMA involves production of the superoxide anion radical. The superoxide anion radical is a strong reducing agent and reduces cytochrome

c, which is detected by an increase in absorbance of the sample. Additionally, these cells produce the nitric oxide radical, which reacts with superoxide anion radical at diffusion-limited rates to produce the strong oxidant, peroxynitrite anion (Ischiropoulos et al., 1992). These strong oxidants apparently cause loss of absorbance of the cytochrome. In our assay, low concentrations of the mimetic peptide (1 nM) or lipopolysaccharide resulted typically in an increase in absorbance of cytochrome *c*. Higher concentrations of peptide (5 to 10 nM) consistently caused rapid loss of absorbance, evidence of destruction of cytochrome *c* (Figure 7A).

Figure 7B shows an experiment in which the number of cells was lowered to allow cytochrome *c* to compete effectively for the reactive superoxide anion when nitric oxide was produced at a lower rate. Without treatment with the peptide, again no reduction of the cytochrome was detected. The rate of reduction of cytochrome *c* correlated with the amount of peptide mimetic added, and the activity of the branched mimetic peptide was more active than the linear peptide. These results are typical of those in the literature with activated macrophages, which were commonly reported as single-point measurements at 10 or 20 min rather than time courses shown in Figure 7. The branched peptide mimetic caused a stronger response than the linear peptide. Absorbance of control samples without stimulant did not change. These results show a strong response by the cells to the branched peptide, even stronger than to lipopolysaccharide, on an equal weight basis, in stimulating the oxidative burst.

The response of cells to the peptide was also assayed by oxidation of pyrogallol (Marklund and Marklund, 1974) in a reaction initiated by superoxide anion radical (Figure 8). The peptide mimetic was the most active stimulant in these experiments, which confirmed activity of the peptide on cells by biochemical assays *in vitro*.

b) Phagocytosis

Phagocytosis is measured by the uptake of fluorescently-labeled bacterial cells (Molecular Probes, Inc.) (Figures 9 and 10) or fluorescent polystyrene beads (Polyscience, Inc) (Figure 11 and 11A). Within 10 to 30 min after addition, microscopic examination showed the presence of these particles within cells. Unstimulated cells exhibited a low level of phagocytosis or did not contain any detectable bacterial cells or beads. Phagocytosis can be quantitated by quenching extracellular (unphagocytized) bacterial cells by addition of the dye trypan blue to the suspension and measuring the remaining (intracellular) fluorescence (Wan et al., 1993). In addition, the peptide mimetic induced detachment of neutrophils from the surface, an indication of the chemotaxis that is primed by the vitamin D-binding protein (Binder et al., 1999). These assays of phagocytic activity allow correlation of activity at the cellular level during the course of treatment of each animal.

c) Pharmacokinetic analysis

The peptide was tagged with a fluorescent dansyl group to follow the life-time of the peptide in blood. Preliminary analyses have shown that a portion of the peptide,

approximately two-thirds, binds to proteins when added to serum. The association of the peptide with proteins may be advantageous to prevent rapid removal from the circulatory system as the result of clearance of small proteins by the kidneys (Goochee et al., 1991). The sensitivity of the fluorescence measurements allows analysis of the peptide concentration to levels 100-fold less than the initial level. This range is sufficient to determine half-life of the peptide *in vivo*.

2. Pre-clinical observations

Owners of dogs that were used to obtain preliminary data were required to sign informed consent and agreement to necropsy forms. Dogs with histologically or cytologically confirmed cancer are given the peptide mimetic at doses of 100 to 1,500 μg by perfusion into the blood or subcutaneously. The effective dose of the glycopeptide used by Schneider et al. (2003) was 0.4 ng/g body weight given every other day. On this basis, a minimal weekly dose should be 1.4 ng/g body weight. For a large animal (80 lb or 36 kg dog), the starting, minimal-effective, weekly dose of the synthetic mimetic peptide would therefore be 50 μg or 6.8 nmoles. For initial studies, the mimetic peptide was administered on a weekly basis at a 10-fold higher dose to test whether any adverse effects are experienced.

Canine patients with spontaneous malignancies were treated with the peptide mimetic. All patients had been treated with chemotherapy but had recurring, advanced cancer at the initiation of treatment with the branched peptide mimetic. Tumor types included lymphoma (n=4), pulmonary metastatic osteosarcoma (n=2), pulmonary metastatic hemangiosarcoma (n=1), pulmonary metastatic fibrosarcoma (n=1), lymphoid leukemia (n=1), pulmonary metastatic chemodectoma (n=1), pulmonary metastatic nerve sheath tumor (n=1), and acanthomatous epulis (n=1*). No adverse clinical events were observed at the highest dose of 200 nmoles administered on a weekly basis.* Eleven patients were treated for measurable disease, including 3 lymphoma patients who relapsed following chemotherapy treatment. One lymphoma patient is currently undergoing treatment concurrently with reinduction of chemotherapy following relapse and was considered to be in remission at the time treatment was initiated. Two patients died before evaluation of response. In these patients, death was attributed to the advanced cancer and not an adverse event to the peptide mimetic. One patient with lymphoma experienced a partial response and a second patient with lymphoma had a minimal response. Subjectively, quality of life was dramatically enhanced with the use of the peptide mimetic and several patients appeared to have extension of life beyond what would be normally expected with their advanced cancer. With several patients, behavior was restored to pre-disease activities. Although the treatment seems to hold great promise, data are limited because of the short period of treatment and small population size.

TABLES

Table 1. Amino acid sequences derived from DNA sequences of phage particles selected with GalNAc-specific lectins.

12-Mer (*H. pomatia* lectin)

1. AQALGLSAISPR
2. CTDEALYTRRQC
3. VQATQSNQHTPR
4. EQATPRNHHSPP
5. VQATPRLQHTPR
6. AQGPPSKQHSPP
7. LPTTINISNRGS
8. VPFRGYSPPQG
9. VQAIQSNQLTPR
10. VQATTVQIQHAP
11. CRASINITNRGS
12. LPSTINITNRGS
13. QSTTINIIRSGS
14. EEAISLISIRRR
15. VQAGQSNAHTAG
16. VQATQSNQHTPR
17. TTDEPFVYRRQP
18. VQARQSNQHTPR
19. VQANQCQSAYAR
20. VRLLQYAHRGRG
21. VQATQSNQHTPR
22. VQNYQSNQHTPR
23. FVSTTMKLSDG
24. FBSYDTEAFGGS

7-Mer flanked by C (*H. pomatia* lectin)

25. CNSTTPASC
26. CDOTESSFC
27. CSPHTKDWC
28. CGPDPPRDC
29. CNWHWITNC
30. CSVSQVTTC
31. CEQTLTPQC
32. CLSPLSPVC
33. CLTSSVSTC
34. CVDIPSFQC
35. CTVSGHQDC
36. CLHPMLTDC
37. CCALDLETC
38. CDSPNHRLC
39. CMTSFNLSC

7-Mer flanked by C (VVA Lectin)

40. CLNNSHAEC
41. CPQNTAKAC
42. CPFRSHQRC
43. CPLLPWSPC
44. CSSIPGPSC
45. CVNTSSDSC
46. CPSRTPNHC
47. CYSHNLAEC
48. CTPPKTRTC
49. CDPMRPSMC
50. CPRLSQSPC
51. CSLDYPDSC

7-Mer (*H. pomatia* lectin)

52. SHVQCVN
53. IPNPSIR
54. RIRVIRE
55. EYDNSPP
56. RTEHAGF
57. YVSDYDW
58. SDRPSLK
59. YWSPSLK
60. LPLKLLW
61. HAHKVGT
62. ALKPMSH
63. TPDYLAA
64. TPPAAAR
65. YPSTFTR
66. VCRPPCP
67. MPLPFPT
68. ASDTIQT
69. SYYMRDP
70. SQDPSQL
71. LQTFPKP
72. LSNTFGL
73. IPWASLL
74. ITANTLS

75. KISLGGL
76. APQPYRQ
77. HSPADTP
78. TLPALAL
79. NAQKSTL
80. ADEALTL
81. SLSASRI
82. GSASALA
83. SNLSGST
84. QVPVHPS
85. IPGTVHV
86. TTTSFRA
87. ATSLVNL
88. ASGMVFM
89. QLFPCMS
90. LITHPIV
91. YTLGDPS
92. LRPMTVP
93. LGTTPQL
94. TAFLGQH
95. YHQRGPV
96. SHLKSMS
97. HMSRMAN
98. ASTQLLP
99. SALWSPV
100. VLEYSPS
101. SQPATKR

7-Mer (VVA lectin)

102. DPKVRTA
103. FERDLPW
104. NRAQNRK
104. AYPFIFR
105. LGILCSR
106. GEYVTLR
107. HLDSSNS
108. LNTARHT
109. TSVLRPG
110. HVPPHAR
111. GPRTHNS
112. QMPAVPS
113. WNPTYPP
114. HQDLRRQ
115. GELPFNP
116. SYLQLPP
117. HVLPVPL
118. ASTYLLG
119. YERAGSH
120. WQPHSHP
121. DSLTPET
122. HPNRFDH
123. NNAILHP
124. RLPGHPS
125. HAPHLWD
126. SPNVPPY
127. IPHLSTL
128. DYPASSF
129. FPRMQPL
120. HNKTSYY
121. THHPIHK
122. TSPLPYW
123. ASPHPAV
124. YSLQHML
125. FPTTYWI
126. CLRAMND
127. NKLPPLF
128. SGLQQPR
129. QATKVRS
130. SPTSARS
131. ASHPSSA
132. QPIGAQR
133. LDTHHLQ
134. QPSLHIS

Table 2. Example of documentation of the algorithm used to identify patterns within the selected amino acid sequences.

PROGRAM DOCUMENTATION

In order to derive the consensus sequence from the sequences obtained from phage display, a code is written in Java language (1.4.1_02), which is a stand-alone program. The program takes all the sequences that are separated by a separator (|) into a single string as an input. This string is converted into substrings and is stored in a vector, and then these substrings are converted into six-letter codes and transforms into triplets.

Example:

Actual sequence is **AQQSQVY|AQQSQAY**

Converted sequence will be **AXXOXUY, AXXOXAY**

Triplets obtained are **AXX XXO XOX OXU XUY|AXX XXO XOX OXA XAY**

Then the program calculates the position, total and frequency of all the triplets and displays in the form of matrix. Total is sum of the individual triplets occurring in all the sequences and Frequency is (Total/Length of the sequence)*100.

Example:

*	1	2	3	4	5	TOT	Freq=(TOT/Len)*100
AXX	2	0	0	0	0	2	20.0%
OXA	0	0	0	1	0	1	10.0%
OXU	0	0	0	1	0	1	10.0%
XAY	0	0	0	0	1	1	10.0%
XOX	0	0	2	0	0	2	20.0%
XUY	0	0	0	0	1	1	10.0%
XXO	0	2	0	0	0	2	20.0%

Triplet that occur more than one time are taken and calculated again considering the amino acid immediately before and after the triplet from the sequence.

Example:

Triplets that are occurring more than one time are: **AXX, XOX, XXO**

Amino acid before and after triplet from the sequence are displayed

Converted: **AXXO**
Original: **AQQS**

Converted: **AXXO**
Original: **AQQS**

Converted: **XXOXU**
Original: **QQSQV**

Converted: **XXOXA**
Original: **QQSQA**

Converted: **AXXOX**
Original: **AQQSQ**

Converted: **AXXOX**
Original: **AQQSQ**

Table 3. Results of the analysis of the sequence data-base with the algorithm illustrated in Table 2 and Figure 1. The Table shows the frequency of each dimer, trimer and pentamer in the collected mimetic sequences. The amino acids were converted to a generic functional designation to expand the flexibility of the analysis.

Results of 12-mer sequences

2-Mers

Seq	Total	Freq
AB	2	0.76%
AG	3	1.14%
AH	2	0.76%
AO	7	2.66%
AP	1	0.38%
AU	5	1.9%
AX	3	1.14%
AY	1	0.38%
AZ	1	0.38%
BA	1	0.38%
BB	4	1.52%
BG	6	2.28%
BO	2	0.76%
BU	3	1.14%
BX	5	1.9%
CB	1	0.38%
CO	1	0.38%
CX	1	0.38%
GB	1	0.38%
GG	1	0.38%
GO	5	1.9%
GP	1	0.38%
GU	1	0.38%
GX	1	0.38%
GY	1	0.38%
HA	1	0.38%
HB	1	0.38%
HH	1	0.38%
HO	9	3.42%
JA	3	1.14%
JG	1	0.38%
JJ	3	1.14%
JO	1	0.38%
JP	1	0.38%
JX	1	0.38%
MB	1	0.38%
OA	3	1.14%
OB	2	0.76%
OG	1	0.38%
OJ	4	1.52%
OM	1	0.38%
OO	8	3.04%
OP	13	4.94%
OU	7	2.66%
OX	13	4.94%
OY	1	0.38%
PB	10	3.8%
PO	3	1.14%
PP	4	1.52%
PX	1	0.38%
PZ	2	0.76%
UB	3	1.14%
UG	1	0.38%
UO	10	3.8%
UP	3	1.14%
UU	3	1.14%
UX	19	7.22%
UY	2	0.76%
XA	13	4.94%
XB	3	1.14%
XC	2	0.76%
XG	1	0.38%
XH	9	3.42%
XO	9	3.42%
XP	1	0.38%
XU	6	2.28%
XX	8	3.04%
XY	2	0.76%
YA	2	0.76%
YB	1	0.38%
YJ	1	0.38%
YO	2	0.76%
YX	1	0.38%
ZB	2	0.76%
ZG	1	0.38%
ZU	2	0.76%

3-Mers

Seq	Total	Freq
AEX	1	0.42%
AGX	1	0.42%
AHB	1	0.42%
AHO	1	0.42%
AOO	1	0.42%
AOP	2	0.84%
AOU	1	0.42%
AOX	3	1.26%
AUG	1	0.42%
AUO	2	0.84%
AUX	1	0.42%
AUY	1	0.42%
AXA	1	0.42%
AXG	1	0.42%
AXX	1	0.42%
AYA	1	0.42%
AZG	1	0.42%
BAO	1	0.42%
BBB	1	0.42%
BEX	2	0.84%
BGB	1	0.42%
BGO	3	1.26%
BGY	1	0.42%
BOG	1	0.42%
BOY	1	0.42%
BUO	1	0.42%
BUU	1	0.42%
BUX	1	0.42%
BXC	1	0.42%
BXH	2	0.84%
BXO	1	0.42%
BXP	1	0.42%
CBA	1	0.42%
COJ	1	0.42%
CXO	1	0.42%
GBG	1	0.42%
GGO	1	0.42%
GPP	1	0.42%
GUO	1	0.42%
GXO	1	0.42%
GYO	1	0.42%
HAP	1	0.42%
HBG	1	0.42%
HHO	1	0.42%
HOA	1	0.42%
HOP	8	3.35%
JAU	2	0.84%
JAZ	1	0.42%
JJA	2	0.84%
JJP	1	0.42%
JOJ	1	0.42%
JPZ	1	0.42%
JXA	1	0.42%
MBU	1	0.42%
OAG	1	0.42%
OAU	1	0.42%
OAY	1	0.42%
OBB	1	0.42%
OBX	1	0.42%
OGO	1	0.42%
OJA	1	0.42%
OJG	1	0.42%
OJJ	2	0.84%
OMB	1	0.42%
OOJ	1	0.42%
OOM	1	0.42%
OOO	2	0.84%
OOU	4	1.67%
OPB	10	4.18%
OPP	3	1.26%
OUB	1	0.42%
OUU	1	0.42%
OUX	5	2.09%
OXA	1	0.42%
OXB	3	1.26%
OXO	3	1.26%
OXX	6	2.51%
OYJ	1	0.42%

5-Mers

Seq	Total	Freq
ABXOX	1	0.52%
AGXOX	1	0.52%
AHBGB	1	0.52%
AHOAG	1	0.52%
AOOUX	1	0.52%
AOPBU	1	0.52%
AOPBX	1	0.52%
AOUXU	1	0.52%
AOXOX	3	1.57%
AUGUO	1	0.52%
AUOPB	1	0.52%
AUOUU	1	0.52%
AUXOX	1	0.52%
AXAUG	1	0.52%
AXGPP	1	0.52%
AXOCX	1	0.52%
AZGGO	1	0.52%
BAOUX	1	0.52%
BGYOP	1	0.52%
BOYJO	1	0.52%
BUOJG	1	0.52%
BUUXY	1	0.52%
BUXHO	1	0.52%
BXHHO	1	0.52%
BXHOP	1	0.52%
BXOXX	1	0.52%
CBAOU	1	0.52%
COJJA	1	0.52%
CXOAY	1	0.52%
GPPOB	1	0.52%
GUOAU	1	0.52%
GXOXA	1	0.52%
GYOPP	1	0.52%
HBGBG	1	0.52%
HHOPP	1	0.52%
JAUOU	1	0.52%
JAUYO	1	0.52%
JAZGG	1	0.52%
JJAUO	1	0.52%
JJAUY	1	0.52%
JJPZU	1	0.52%
JOJAZ	1	0.52%
JPZUY	1	0.52%
JXAOP	1	0.52%
MBUOJ	1	0.52%
OAUOP	1	0.52%
OAYAB	1	0.52%
OBBXC	1	0.52%
OBXHO	1	0.52%
OJAZG	1	0.52%
OJJAU	1	0.52%
OJJPZ	1	0.52%
OMBUO	1	0.52%
OOJJP	1	0.52%
OOMBU	1	0.52%
OOOMB	1	0.52%
OOOUX	1	0.52%
OOUXU	4	2.09%
OPBUX	1	0.52%
OPBXH	1	0.52%
OPPXA	1	0.52%
OUBBB	1	0.52%
OUBUO	1	0.52%
OUXUO	3	1.57%
OUXUU	1	0.52%
OUXUX	1	0.52%
OXAHO	1	0.52%
OXBOO	3	1.57%
OXXHO	5	2.62%
OXXUO	1	0.52%
OYJOJ	1	0.52%
PBUXH	1	0.52%
PBXHH	1	0.52%
POBXH	1	0.52%
POOUX	2	1.05%
PPOBX	1	0.52%

3-Mers

Seq	Total	Freq
PBU	1	0.42%
PBX	1	0.42%
POB	1	0.42%
POO	2	0.84%
PPO	1	0.42%
PPX	1	0.42%
PXA	1	0.42%
PZB	1	0.42%
PZU	1	0.42%
UBB	1	0.42%
UBO	1	0.42%
UBU	1	0.42%
UGU	1	0.42%
UOA	1	0.42%
UOJ	1	0.42%
UOO	1	0.42%
UOP	2	0.84%
UOU	2	0.84%
UOX	3	1.26%
UPO	2	0.84%
UPZ	1	0.42%
UUB	1	0.42%
UUO	1	0.42%
UUX	1	0.42%
UXA	9	3.77%
UXH	2	0.84%
UXO	1	0.42%
UXU	5	2.09%
UXX	1	0.42%
UXY	1	0.42%
UYB	1	0.42%
UYO	1	0.42%
XAB	1	0.42%
XAG	2	0.84%
XAH	1	0.42%
XAO	6	2.51%
XAU	2	0.84%
XAX	1	0.42%
XBG	3	1.26%
XCX	1	0.42%
XGP	1	0.42%
XHA	1	0.42%
XHH	1	0.42%
XHO	7	2.93%
XOA	1	0.42%
XOO	1	0.42%
XOX	7	2.93%
XUO	4	1.67%
XUU	1	0.42%
XUX	1	0.42%
XXC	1	0.42%
XXH	5	2.09%
XXU	1	0.42%
XXY	1	0.42%
XYA	1	0.42%
XYX	1	0.42%
YAB	1	0.42%
YAH	1	0.42%
YBB	1	0.42%
YJO	1	0.42%
YOB	1	0.42%
YOP	1	0.42%
YXO	1	0.42%
ZBG	1	0.42%
ZBO	1	0.42%
ZGG	1	0.42%
ZUO	1	0.42%
ZUY	1	0.42%

5-Mers

Seq	Total	Freq
PPXAG	1	0.52%
PZBGY	1	0.52%
PZUYB	1	0.52%
UBOGO	1	0.52%
UBUUX	1	0.52%
UGUOA	1	0.52%
UOAUO	1	0.52%
UOOOM	1	0.52%
UOUBB	1	0.52%
UOUUO	1	0.52%
UOXBG	3	1.57%
UPOOU	2	1.05%
UPZBG	1	0.52%
UUBOG	1	0.52%
UUOUB	1	0.52%
UUXYA	1	0.52%
UXABX	1	0.52%
UXAGX	1	0.52%
UXAOO	1	0.52%
UXAOP	1	0.52%
UXAOX	3	1.57%
UXAUX	1	0.52%
UXAXX	1	0.52%
UXHAP	1	0.52%
UXHOP	1	0.52%
UXOXX	1	0.52%
UXUOX	3	1.57%
UXUUB	1	0.52%
UXUXH	1	0.52%
UXXYX	1	0.52%
UXYAH	1	0.52%
UYBBX	1	0.52%
UYOBB	1	0.52%
XABXO	1	0.52%
XAGXO	1	0.52%
XAHOA	1	0.52%
XAOOU	1	0.52%
XAOPB	2	1.05%
XAOXO	3	1.57%
XAUGU	1	0.52%
XAUXO	1	0.52%
XAXXC	1	0.52%
XCXOA	1	0.52%
XGPPO	1	0.52%
XHHOP	1	0.52%
XHOPB	6	3.14%
XHOPP	1	0.52%
XOAYA	1	0.52%
XOOOU	1	0.52%
XOXAH	1	0.52%
XOXXH	5	2.62%
XOXXU	1	0.52%
XUOPB	1	0.52%
XUOXB	3	1.57%
XUUBO	1	0.52%
XUXHA	1	0.52%
XXCXO	1	0.52%
XXHOP	5	2.62%
XXUOP	1	0.52%
XXYXO	1	0.52%
XYAHB	1	0.52%
XYXOX	1	0.52%
YAHBG	1	0.52%
YBBXP	1	0.52%
YJOJA	1	0.52%
YOBBX	1	0.52%
YOPPX	1	0.52%
YXOXX	1	0.52%
ZBGYO	1	0.52%
ZBOYJ	1	0.52%
ZUOOO	1	0.52%
ZUYBB	1	0.52%

FIGURES

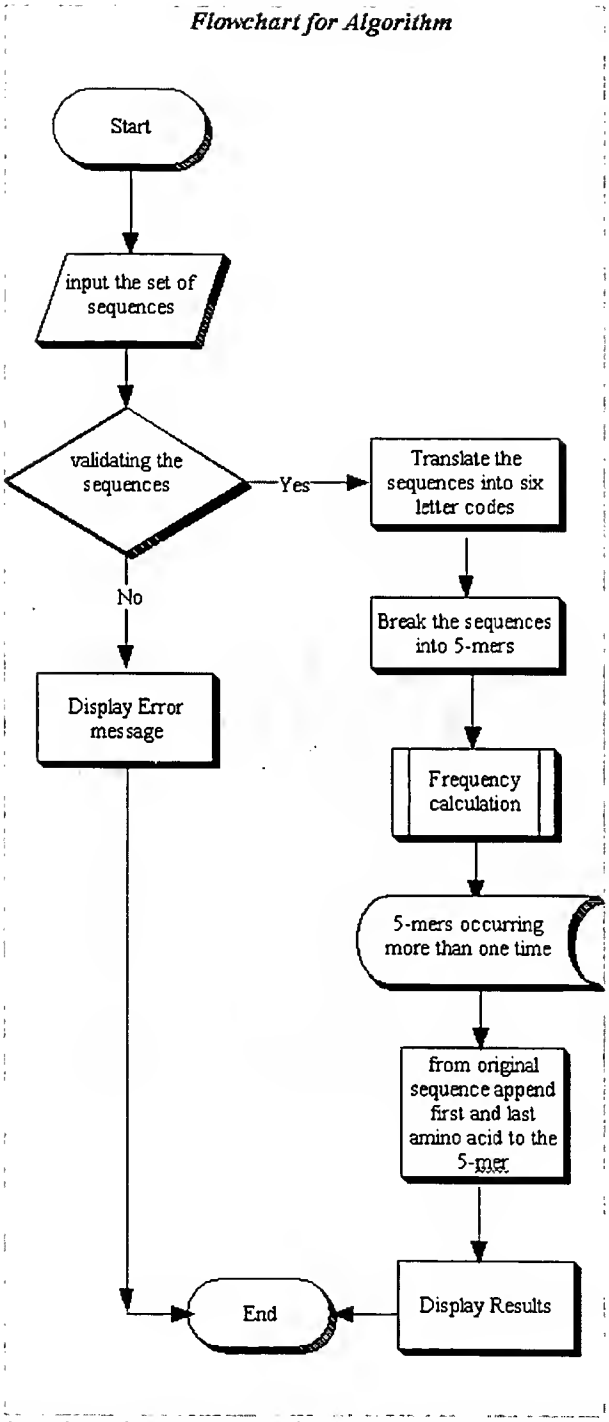

Figure 1. Flowchart that describes the algorithm used to identify patterns in amino acid sequences generated by lectin screens and described in Table 2.

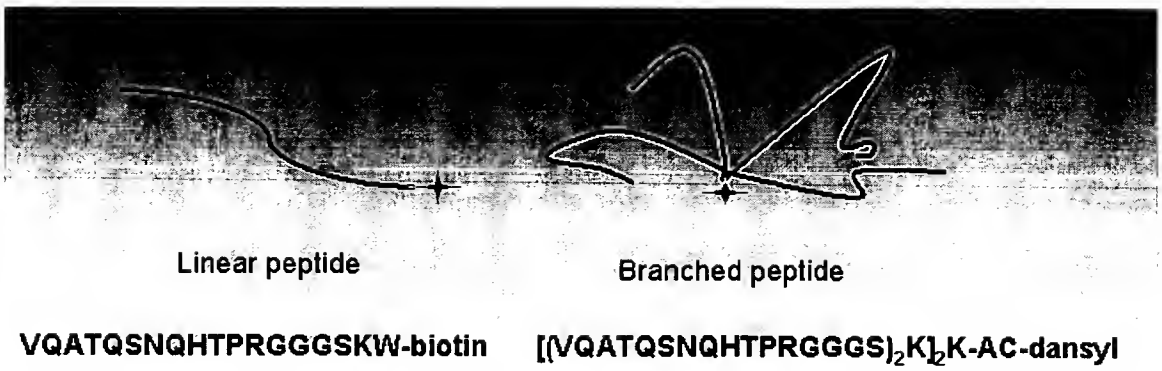

Figure 2. Diagrammatic representations and amino acid sequences of the linear and branched peptide mimetic structures. The star symbols indicate the position of the reporter residue at the C-terminus of the structures.

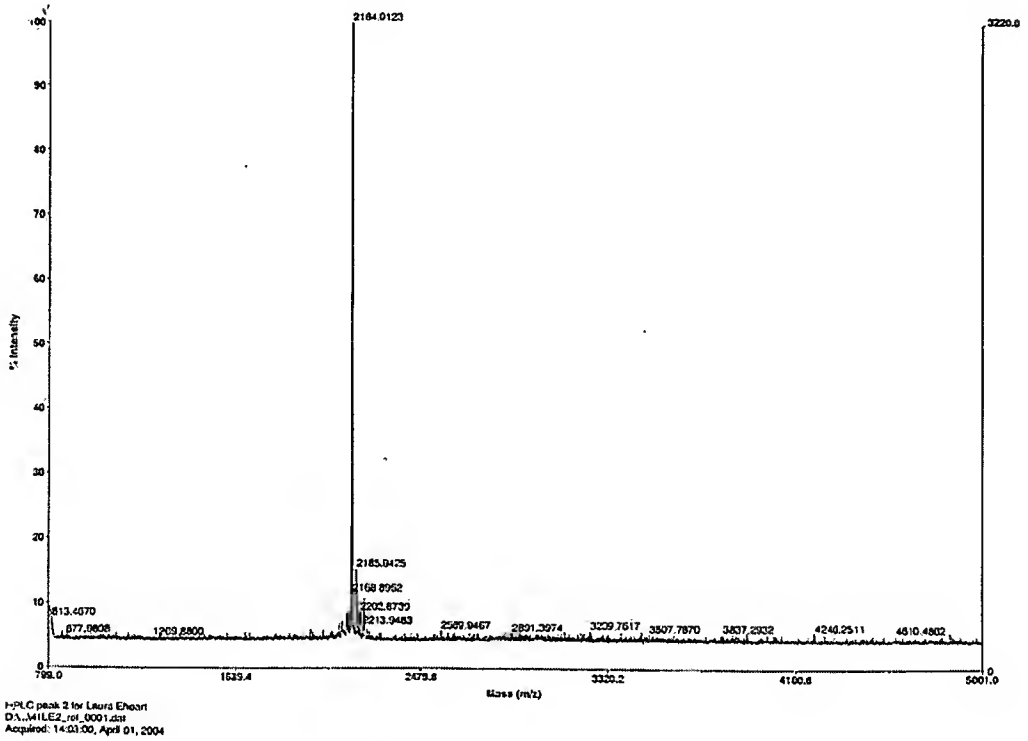

Figure 3. Mass spectroscopic analysis of the linear peptide mimetic shown in Figure 2. The analysis provided a molecular mass of 2,165 Daltons, which is the same as the predicted molecular mass of 2,165 Daltons.

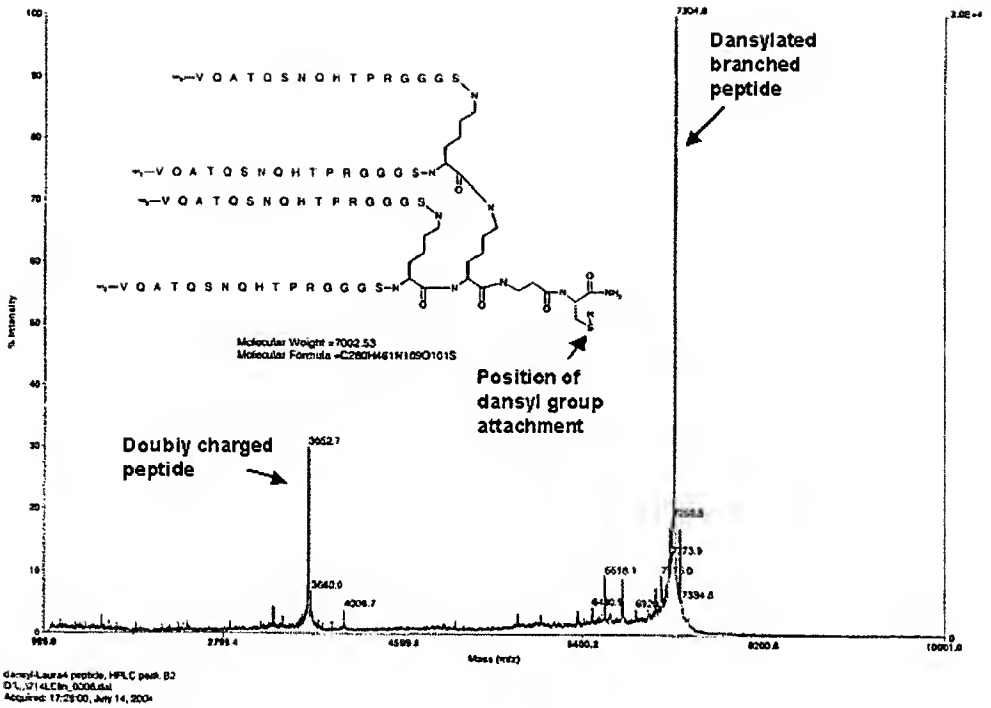

Figure 4. Mass spectrometric analysis of the dansylated branched peptide mimetic structure, with a calculated mass of 7,308 Daltons. The spectrum shows analysis of purified peptide (7,304 to 7,312 Daltons in multiple runs) and doubly charged peptide, 3,653 Daltons (mass/charge ratio). The structure of the undansylated peptide is shown in the figure.

5-((((2-iodoacetyl)amino)ethyl)amino)naphthalene-1-sulfonic acid (1,5-IAEDANS)

Molecular Formula: $C_{14}H_{15}IN_2O_4S$

Molecular Weight: 434.25

CAS Number/Name: 36930-63-9 / 1-Naphthalenesulfonic acid, 5-((2-((iodoacetyl)amino)ethyl)amino)-

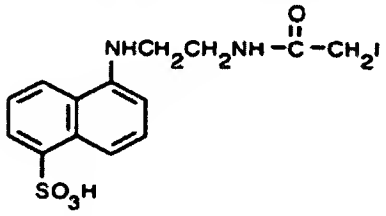

Figure 5. Structure of the dansyl derivative attached to the peptide mimetic shown in Figure 4. Addition occurs at the C-terminal cysteine residue by displacement of the iodine atom on the dansyl derivative by the sulfhydryl sulfur atom in the peptide.

5'3' Frame 1
Met L C A D Y S E N T F T E Y K K K L A E R L K R K L P **Q A T Q S N Q H T**
P R V V V S E A T P T E L A K L V N K R S D F A S N C C S I N S P D L Y C D
S E I D A L L K N I L H H H H H H **Stop**

Figure 6. Predicted amino sequence of a synthetic mimetic gene for DomainIII of Gc-MAF, containing 91 amino acids. The mimetic sequence, which was inserted at the site occupied by GalNAc in Gc-MAF, is highlighted.

Because the N-terminal valine of the consensus sequence (X1-QATQSNQHTPR, wherein X1 is absent or is V) does not seem to be required for mimetic activity (see Table 1), the terminal V was replaced with the N-terminal region of Domain III. Likewise, we replaced the spacer region (GGGS) discussed above with the C-terminal region of DomainIII.

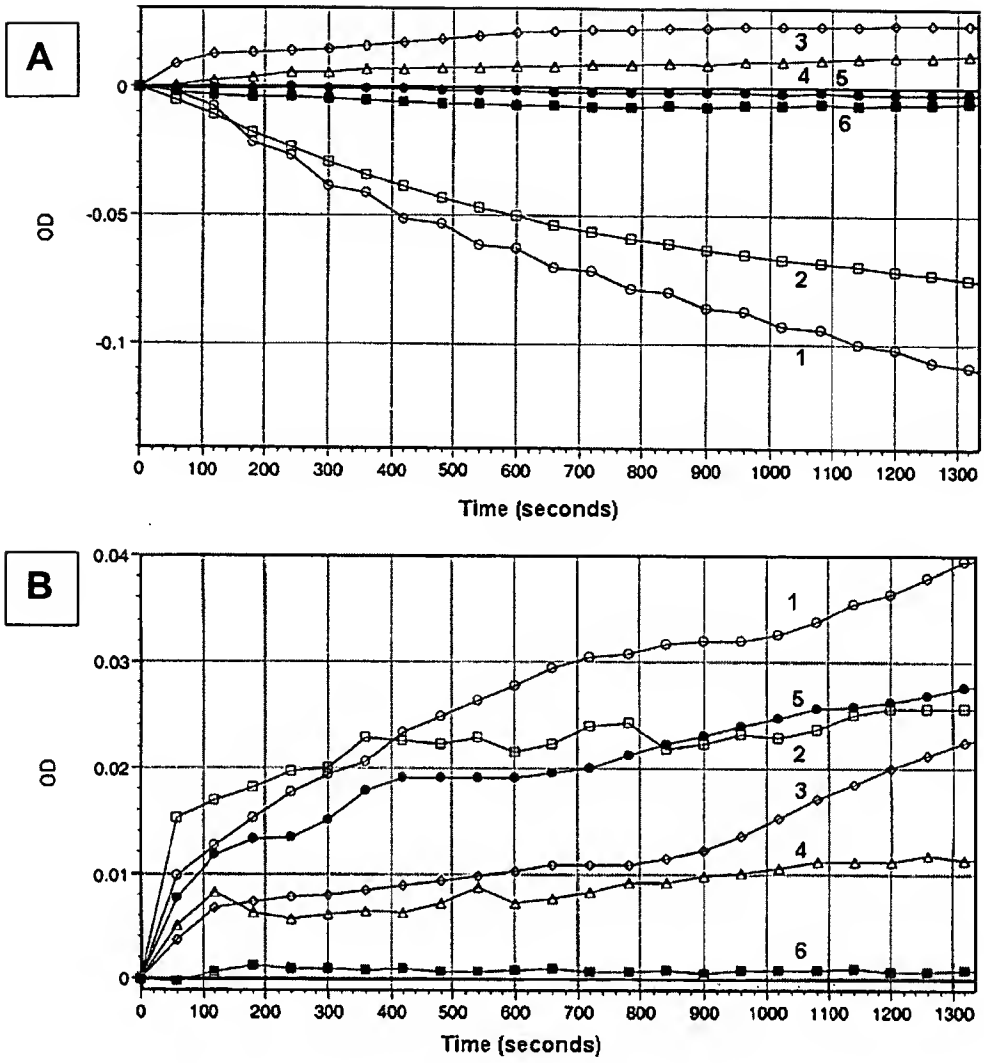

Figure 7. Response of adherent peripheral blood cells to mimetic peptides. Reduction of cytochrome *c* by superoxide anion radical is indicated by an increase in absorbance (OD), whereas a loss of absorbance indicates destruction of the cytochrome. **A**, Total adherent cells from 300 µl of blood were assayed. Samples 1 and 2 contained 5 nM and 2.5 nM branched mimetic peptide, respectively. Samples 3 and 4 contained 5 nM and 2.5 nM linear mimetic peptide, respectively. Sample 5 contained 50 ng/ml lipopolysaccharide (weight equivalent to 6 nM peptide). Sample 6, untreated control cells. **B**, Adherent cells were scraped from the surface and 1 X 10^5 cells were placed in each well. Samples 1 and 2 contained 10 nM and 5 nM mimetic peptide, respectively. Samples 3 and 4 contained 10 nM and 5 nM linear peptide, respectively. Sample 5 contained 50 ng/ml lipopolysaccharide. Sample 6, untreated control cells.

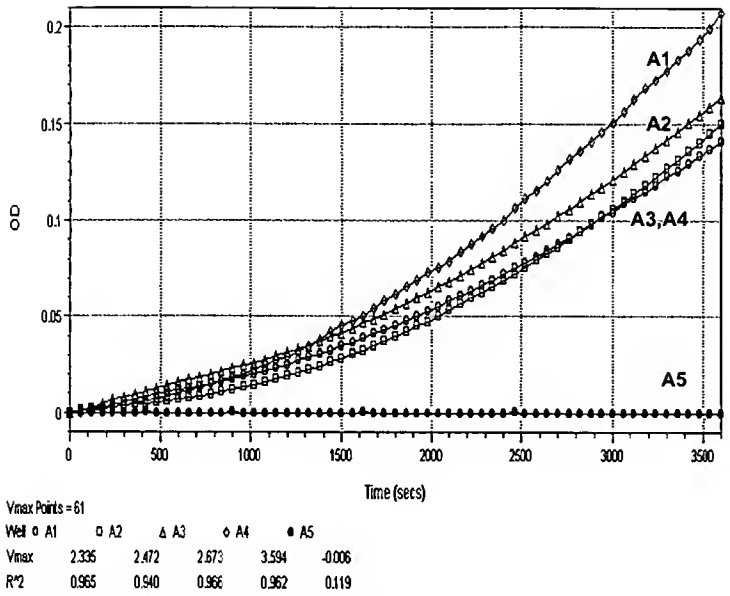

Figure 8. Assay of pyrogallol oxidation initiated by superoxide anion radical generation by peripheral blood adherent cells treated with stimulants. A1, A2, A3: The branched peptide mimetic was added to 3.4, 1.7 or 0.7 nM (25, 12.5 or 5 ng/ml). A4: 50 ng/ml lipopolysaccharide. A5: No treatment.

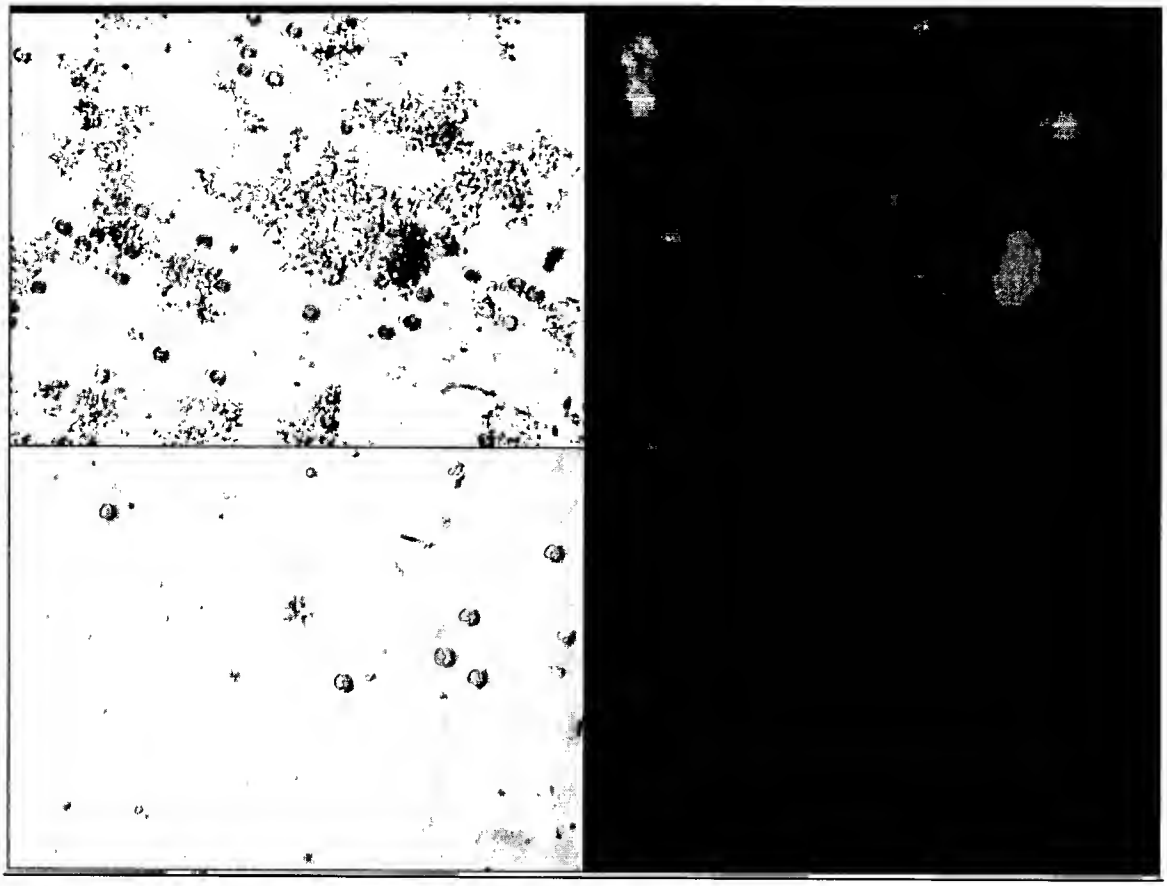

Figure 9. Microscopic analysis of phagocytosis of fluorescently labeled bacterial cells by peripheral blood macrophages. **Upper panels:** The right upper panel shows a fluorescent image of the light microscopic image shown on the left. The sample was treated 15 h with 5 nM branched peptide and then incubated with bacterial cells for 30 min. Fluorescence of bacterial cells that remained extracellular was quenched with trypan blue. **Lower panels:** The right lower panel shows a fluorescent image of cells in the left lower panel that were not treated with the peptide. Such control samples show the highest level of phagocytosis of bacterial that we have observed. In many experiments, control cells show no fluorescence.

Figure 10. A higher magnification of macrophages after phagocytosis of fluorescently labeled bacterial cells. Trypan blue was added before microscopic examination to quench fluorescence of extracellular bacteria, two of which are marked by arrows.

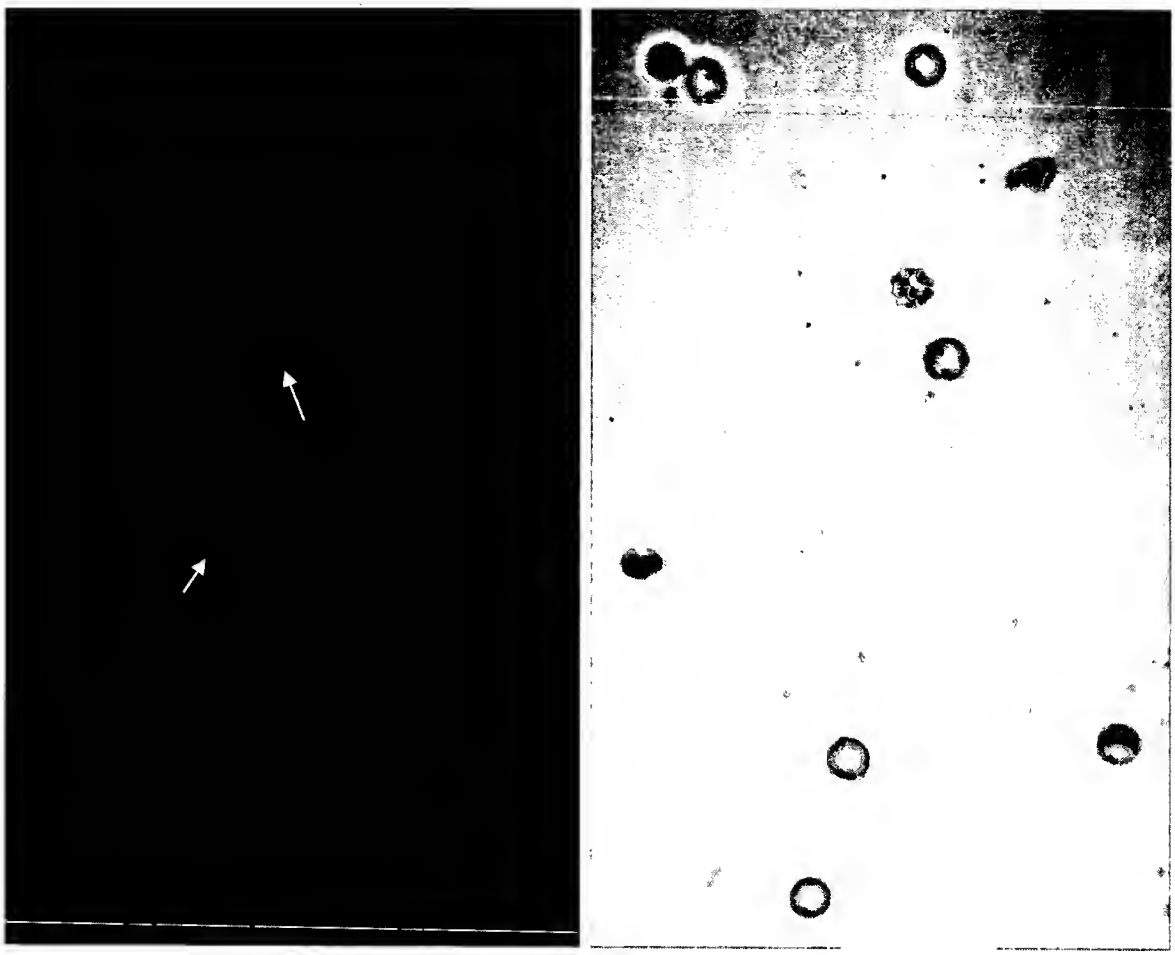

Figure 11. Phagocytosis of fluorescent beads. *Left,* Fluorescence microscopy image. R*ight*, phase microscopy image of the left-hand panel. Extracellular beads appear as dark particles on the right-hand phase-contrast image, and as small fluorescenct particles in the left-hand panel (indicated by arrows). Cells are not fluorescent unless beads are engulfed. The branched peptide mimetic was added at 3.4 nM (25 ng/ml).

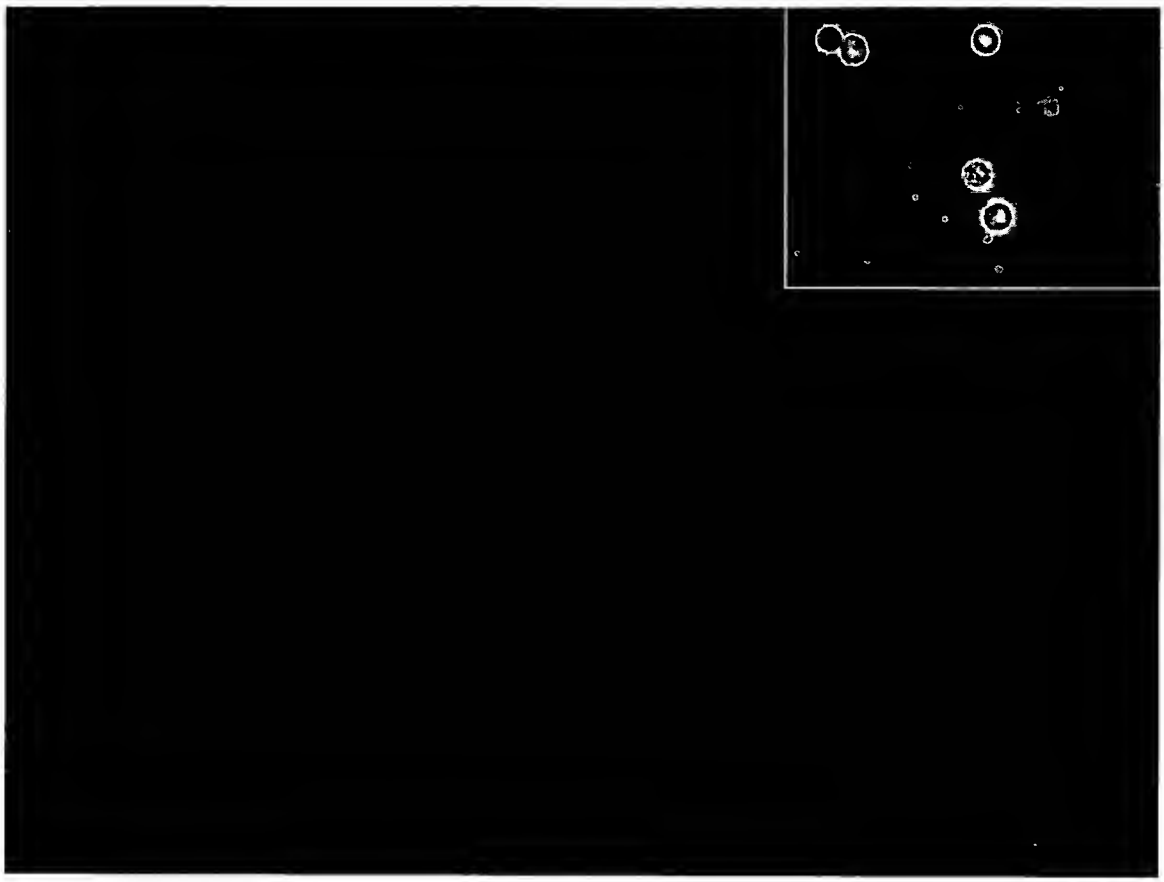

Figure 11A. Enlarged portion of Figure 11.

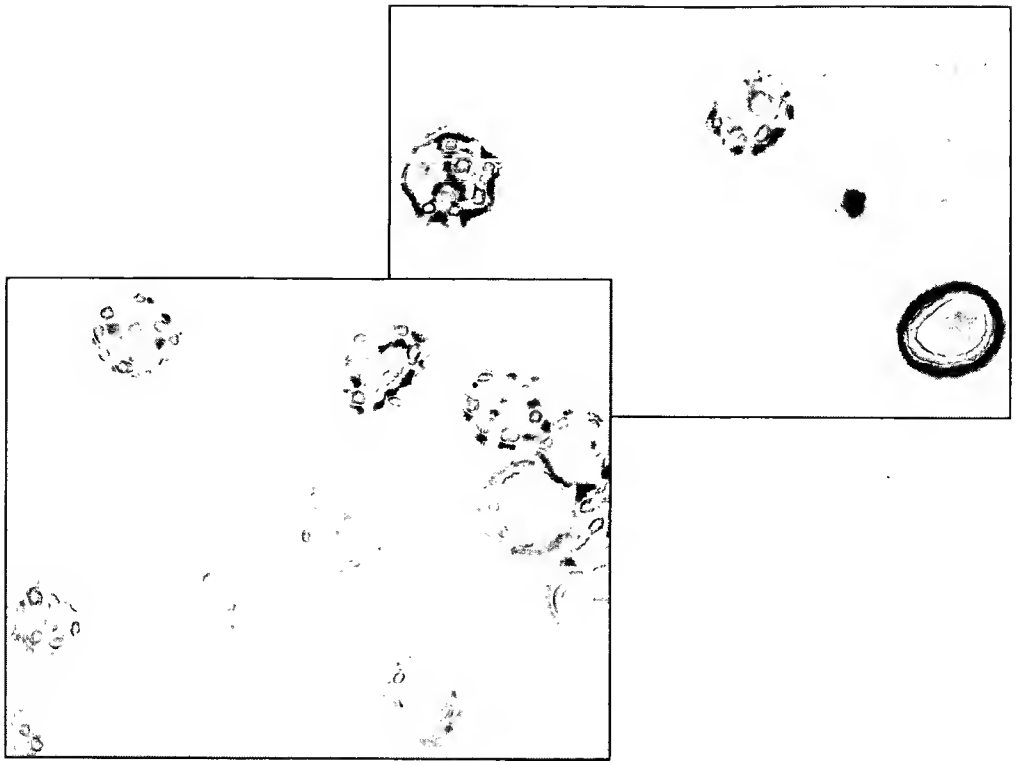

Figure 12. Phagocytosis of fluorescent polystyrene beads by adherent cells of peripheral blood. The branched peptide mimetic was added at 3.4 nM (25 ng/ml). Unstimulated cells did not contain beads (not shown).

Application Data Sheet

Application Information

Application Type::	Provisional
Subject Matter::	Utility
Suggested Classification::	N/A
Suggested Group Art Unit::	N/A
CD-Rom or CD-R?	None
Title::	Immunostimulation Through Activation of Phagocytic Cells
Attorney Docket Number::	04-997
Request for Early Publication?::	No
Request for Non-Publication?::	No
Suggested Drawing Figure::	N/A
Total Drawing Sheets::	11
Small Entity::	Yes
Petition Included?::	No
Secrecy Order in Parent Appl.?::	No

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